THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 22, Issue of June 3, pp. 21661–21666, 2005
Printed in U.S.A.


# The Small G-protein $Arf6_{GTP}$ Recruits the AP-2 Adaptor Complex to Membranes*

Received for publication, March 21, 2005, and in revised form, March 29, 2005
Published, JBC Papers in Press, March 31, 2005, DOI 10.1074/jbc.M503099200

**Olivia Paleotti‡§, Eric Macia§¶, Frederic Luton‡, Stephanie Klein‡, Mariagrazia Partisani‡, Pierre Chardin‡, Tom Kirchhausen¶, and Michel Franco‡‖**

*From the ‡Institut de Pharmacologie Moleculaire et Cellulaire, CNRS-Unité Mixte de Recherche 6097, 660 Route des Lucioles, 06560 Valbonne Sophia-Antipolis, France and ¶Department of Cell Biology, Harvard Medical School and The Center for Blood Research, Institute for Biomedical Research, Boston, Massachusetts 02115*

**The small GTP-binding protein ADP-ribosylation factor 6 (Arf6) is involved in plasma membrane/endosomes trafficking. However, precisely how the activation of Arf6 regulates vesicular transport is still unclear. Here, we show that, *in vitro*, recombinant Arf6GTP recruits purified clathrin-adaptor complex AP-2 (but not AP-1) onto phospholipid liposomes in the absence of phosphoinositides. We also show that phosphoinositides and Arf6 tightly cooperate to translocate AP-2 to the membrane. *In vivo*, Arf6GTP (but not Arf6GDP) was found associated to AP-2. The expression of the GTP-locked mutant of Arf6 leads to the plasma membrane redistribution of AP-2 in Arf6GTP-enriched areas. Finally, we demonstrated that the expression of the GTP-locked mutant of Arf6 inhibits transferrin receptor internalization without affecting its recycling. Altogether, our results demonstrated that Arf6GTP interacts specifically with AP-2 and promotes its membrane recruitment. These findings strongly suggest that Arf6 plays a major role in clathrin-mediated endocytosis by directly controlling the assembly of the AP-2/clathrin coat.**

The ADP-ribosylation factor (Arf)[1] family, which includes six isoforms, regulates vesicular trafficking (1). Arf1, the most studied and abundant isoform, acts mainly at the level of the Golgi complex where it controls the formation of both COPI- and clathrin-coated vesicles. Indeed, Arf1 is required for recruitment of COPI (coatomer) complex and the clathrin adaptors AP-1, AP-3, and AP-4 onto Golgi membranes (2).

Arf6, the most distant isoform, has been involved in plasma membrane/endosome trafficking and cortical actin reorganization (3). Several studies have implicated Arf6 in the regulation of both clathrin-dependent and -independent endocytosis (4–7). Arf6 has also been implicated in recycling and exocytotic events (8–10). Despite a high degree of homology between Arf1 and Arf6 and a similar GTP-dependent membrane association, a direct demonstration of Arf6 involvement in coat recruitment has not yet been obtained. However, it has been recently shown that Arf6, by activation of PI4P5Kinase, could stimulate the membrane association of the clathrin adaptor AP-2 (11). This Arf6-stimulated production of phosphatidylinositol bisphosphate (PIP2) has also been involved in calcium-dependent exocytosis (12). Thus, although it is well established that Arf6 participates in membrane trafficking, its precise role remains obscure. Recently, we have shown that the GDP/GTP cycle of Arf6 occurs mainly at the plasma membrane, suggesting that this is where Arf6 functions take place (13).

AP complexes are heterotetrameric proteins that participate in the formation of coated vesicles as well as the selection of cargo molecules (14, 15). AP-2 is composed of two large ($\alpha$ and $\beta$2, ~100 kDa) one medium ($\mu$2, 50 kDa), and one small ($\sigma$2, 17 kDa) subunits. It is involved in clathrin-coated vesicle formation at the plasma membrane by directly linking the clathrin coat with cargo proteins (16, 17).

In this study, we looked for a possible interaction between Arf6 and the clathrin-adaptor AP-2 complex. We demonstrated that both *in vitro* and *in vivo* Arf6 interacts with AP-2 in a GTP-dependent manner but not with AP-1. We also found that phosphoinositides and Arf6GTP strongly cooperate to recruit AP-2 to the membrane. We then studied the role of Arf6 in clathrin-mediated transferrin receptor endocytosis. We demonstrated that Arf6 is involved in the early stages of clathrin-mediated receptor internalization. Our results suggest that Arf6 is involved in the clathrin-mediated receptor internalization by directly controlling AP-2 assembly onto the plasma membrane.

## EXPERIMENTAL PROCEDURES

*Materials*—Azolectin, egg phosphatidylcholine (PC), liver phosphatidylethanolamine (PE), brain phosphatidylserine (PS) were purchased from Sigma. Brain phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) was from Avanti Polar Lipids (Birmingham, AL). Brain phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol 3,4,5-triphosphate ($PI(3,4,5)P_3$) were from Echelon (Salt Lake City, UT). Transferrin (Tfn) unlabeled nucleotides were from Sigma. Sypro-Orange gel protein staining was from Bio-Rad. 0.4-$\mu$m pore size polycarbonate (Isopore) was from Millipore. $^{125}I$ was from Amersham Biosciences AB (Uppsala, Sweden).

*Cell Culture and Antibodies*—HeLa cell lines stably transfected with Arf6Q67L-HA protein under a tetracycline-regulated inducible system were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal calf serum and antibiotics, and in the presence or absence of doxycycline (Dox) (20 ng/ml). Growing cells in the absence of

* This work was supported by grants from the Association pour la Recherche contre le Cancer and from Groupement des Entreprises pour Lutter contre le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors have contributed equally to this work.
‖ To whom correspondence should be addressed: Institut de Pharmacologie Moleculaire et Cellulaire, CNRS-UMR 6097, 660 Route des Lucioles, 06560 Valbonne-Sophia-Antipolis, France. Tel.: 334-9395-7770; Fax: 334-9395-7710; E-mail: franco@ipmc.cnrs.fr.

[1] The abbreviations used are: Arf, ADP-ribosylation factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PIP2, phosphatidylinositol bisphosphate; PI(3)P, phosphatidylinositol 3-phosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; $PI(3,4)P_2$, phosphatidylinositol 3,4-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-triphosphate; HA hemagglutinin; Dox, doxycycline; BHK, baby hamster kidney; mAb, monoclonal antibody; cpm, cycles/min; MEM, minimal essential medium; BSA, bovine serum albumin; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-3-*O*-(thio)-triphosphate; Tfn, transferrin; Tfn-R, transferrin receptor.

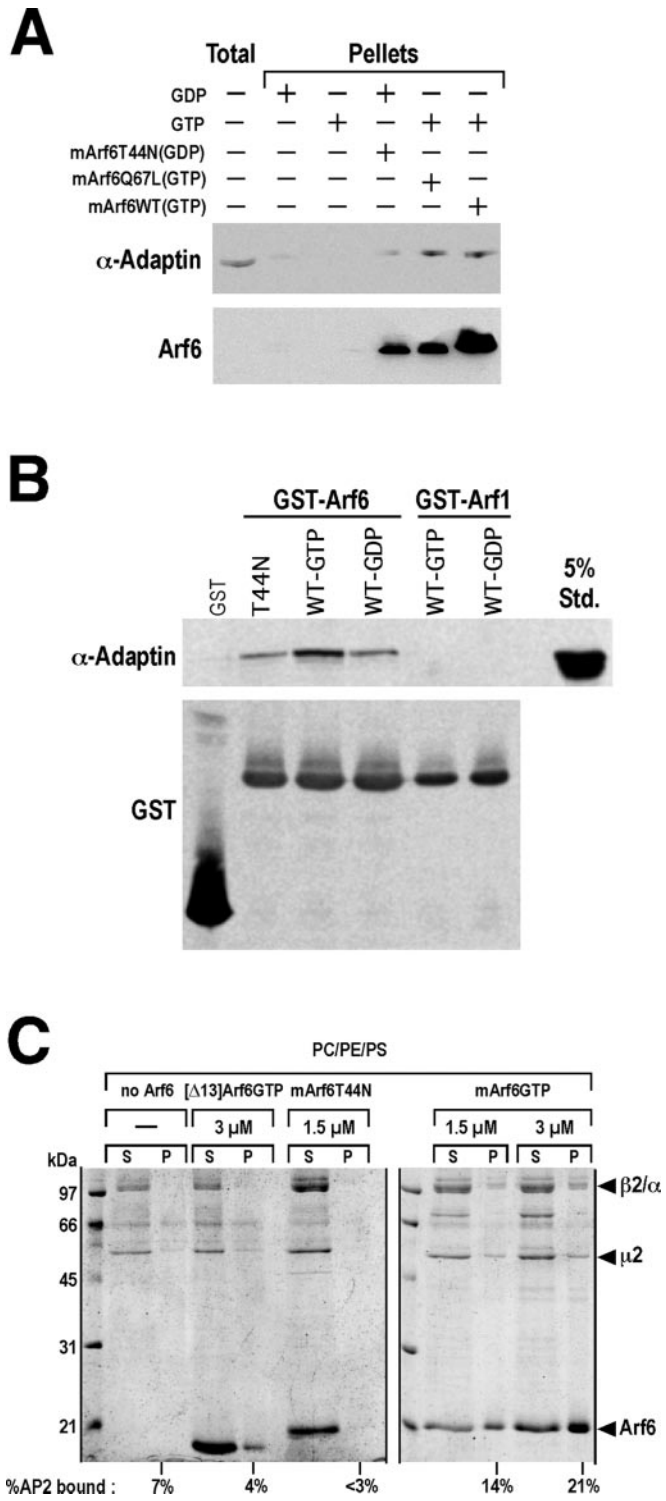


FIG. 1. **Arf6GTP recruits AP-2 to liposomes.** *A*, azolectin liposomes (1 mg/ml) were incubated with brain cytosol (0.5 mg/ml) in the absence or presence of 1.5 μM recombinant myristoylated GDP-locked (*mArf6T44N*) or GTP-locked (*mArf6Q67L*) Arf6 mutants or GTP-loaded wild-type Arf6 (*mArf6WT*) and 1 mM GDP or GTP. After centrifugation, AP-2 and Arf6 bound to the liposomes were analyzed by SDS-PAGE and immunoblotting. *B*, GST or GST-Arf proteins (3 μM) first loaded with GDP (*WT-GDP*) or GTPγS (*WT-GTP*) or locked in GDP (Arf6*T44N*) and bound to glutathione beads were incubated with a detergent extract of rat brain. AP-2 pulled down was analyzed by SDS-PAGE and immunoblotting using anti-α-adaptin antibody. *5% Std.*, 5% of the extract used for affinity purification. The presence of the GST-fused proteins was verified by Western blotting using anti-GST polyclonal antibodies. *C*, the purified AP-2 complex was incubated with recombinant myristoylated GTP-loaded Arf6 wild-type (*mArf6GTP*), Arf6T44N locked in GDP (*mArf6T44N*), and the amino-terminally deleted (Δ13)Arf6GTP in the presence of PC/PE/PS liposomes as described under "Experimental Procedures." After centrifugation, the proportion of proteins recovered in the lipid pellet (*P*) and the supernatant (*S*) was estimated after SDS-PAGE and Sypro-Orange staining. These data were reproduced over three independent experiments.

Dox for 48 h caused a 3–10-fold increased expression of recombinant Arf6 over endogenous proteins, as judged by immunoblot analyses. Baby hamster kidney (BHK) cells were grown in BHK-21 medium (Invitrogen) containing 5% fetal calf serum, 10% tryptose phosphate broth, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The following antibodies were used: rat and mouse monoclonal antibodies (mAbs) specific for HA epitope (clone 3F10 and 12CA5, (Roche Diagnostics GmbH, Mannheim, Germany), mouse mAb against Myc epitope (clone 9E10 Roche Diagnostics), mouse mAb against the transferring receptor (Tfn-R) (clone H68.4 Zymed Laboratories Inc., CA), and mouse mAb against Arf6 (provided by S. Bourgoin, Sainte-Foy, Canada). Mouse mAb against α-adaptin and γ-adaptin (BD Biosciences,) fluorescein isothiocyanate, and Texas-Red-conjugated antibodies were from Jackson ImmunoResearch.

*Confocal Immunofluorescence Microscopy*—BHK cells plated on 11-mm round glass coverslips were transiently transfected with pcDNA3 HA-tagged Arf6 constructs using the FuGENE 6 transfection reagent as described by the manufacturer (Roche Applied Science). The cells were washed twice in phosphate-buffered saline 24 h after transfection and then fixed in 3% paraformaldehyde and processed for immunofluorescence analysis as described previously (18). Confocal microscopy analysis was carried out with a Leica TCS-SP microscope equipped with a mixed gas argon/krypton laser (Leica Microsystems).

*Measurement of Tfn Uptake and Recycling*—Iron-saturated human Tfn was iodinated to a specific activity of ~$10.10^6$ cpm/μg using ICl as described previously (19). For internalization, radiolabeled Tfn ([$^{125}$I]Tfn) ($2.10^6$ cpm/sample) was bound to HeLa cells ($0.5 \times 10^6$ cells/sample) on ice for 2 h in MEM containing 20 mM Hepes/NaOH, pH7.4, and 0.6% bovine serum albumin (MEM/BSA). The cells were washed twice in cold MEM/BSA to remove unbound [$^{125}$I]Tfn and then incubated at 37 or 25 °C, as indicated, to allow internalization. At the indicated times, the cells were rapidly cooled on ice. [$^{125}$I]Tfn was stripped from the cell surface by incubating the cells for 3 min at 4 °C with 100 mM glycine-HCl, pH 2.6, 150 mM NaCl. The cells were then lysed with lysis buffer (20 mM Hepes/NaOH, pH7.4, 1% Triton X-100, 150 mM NaCl) to determine the intracellular [$^{125}$I]Tfn. The total [$^{125}$I]Tfn initially bound to the cells includes the acid-stripped ligand from the cell surface and the cell-associated ligand not sensitive to acid treatment (endocytosed). The values are given as a percentage of the total [$^{125}$I]Tfn. For recycling, cells were incubated for 10 min at 37 °C with [$^{125}$I]Tfn ($2.10^6$ cpm/sample) in MEM/BSA and then cooled down on ice to stop endocytosis. The cells were washed, and surface-bound [$^{125}$I]Tfn was removed by incubating the cells for 2 min at 4 °C with 100 mM glycine/HCl, pH 2.6, and 150 mM NaCl. After three washes in ice-cold MEM/BSA, the cells were further incubated at 25 °C to allow for Tfn recycling. At the indicated times, the medium was collected and replaced by a fresh one. After the last time point, the cells were lysed to recover the intracellular [$^{125}$I]Tfn. The total [$^{125}$I]Tfn includes ligand recovered from the medium (recycled) and cell-associated ligand. Measurements were plotted as a percentage of the total [$^{125}$I]Tfn. The percentage of the spontaneous release that occurred between 0 and 30 s of the non-efficiently stripped plasma membrane [$^{125}$I]Tfn (~10% of the total) was substracted from each time point. Error bars represent the range in duplicate experiments. Each experiment was repeated at least three times with similar results.

*Co-immunoprecipitation Experiments*—BHK-21 cells ($2 \times 10^6$cells in 100-mm tissue culture dishes) were transfected with *myc*-Arf6Q67L or *myc*-Arf6T44N constructs. 24 h after transfection, the cells were lysed in 0.5 ml of lysis buffer (20 mM Hepes/NaOH, pH 7.4, 1% Nonidet P-40, 100 mM NaCl, 1 mM $MgCl_2$, 0.25 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors (Roche Applied Science). Lysates were clarified by centrifugation at 13,000 × g for 30 min; aliquots of each supernatant were kept for analysis. Thereafter, 5 μg of 9E10 antibody and 20 μl of protein A-Sepharose CL4B were added to the clarified lysates for 4 h at 4 °C. The beads were then washed in lysis buffer, and the immunoprecipitated proteins were separated on 12–15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes.

*GST Pull-down Experiments*—GST, GST-Arf6, or GST-Arf1 (3 μM) loaded with 0.1 mM GDP or GTPγS at 1 μM free $Mg^{2+}$ for 30 min at 37 °C were bound to glutathione beads and incubated with 3 mg/ml rat brain extract (in 20 mM Hepes/NaOH, pH7,4, 160 mM sucrose, 1 mM

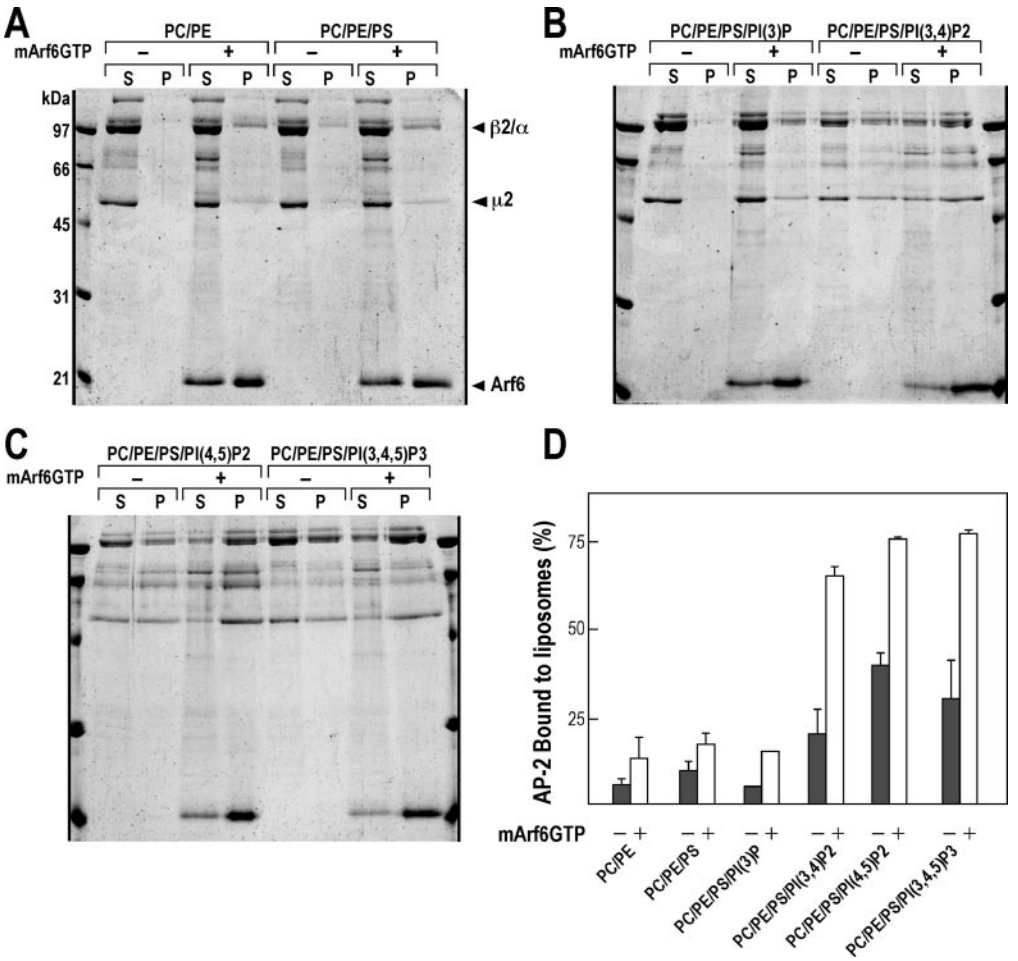


FIG. 2. **Arf6GTP and phosphoinositides cooperate to recruit AP-2 to liposomes.** *A–C*, The purified AP-2 complex (0.4 $\mu$M) was incubated with or without recombinant myristoylated GTP-loaded Arf6 (*mArf6GTP*) (1.5 $\mu$M) in the presence of PC/PE liposomes (1 mg/ml), supplemented or not with PS (*A*) and different phosphoinositides (*B* and *C*). After centrifugation, the proteins recovered in the lipid pellet (*P*) and the supernatant (*S*) were analyzed by SDS-PAGE and Sypro-Orange staining. *D*, representation of the quantification of AP-2 bound to the liposomes in the different conditions tested in *A–C*. *Bars* represent the fraction of AP-2 sedimented in the lipid vesicle pellet as determined by densitometric analysis of the gels and expressed as the percent of the total protein in the assay.

$MgCl_2$, 1% Triton X-100, 1 mM dithiothreitol, and a mixture of protease inhibitors) for 4 h at 4 °C. Thereafter, the beads were washed twice and eluted with 30 $\mu$l of Laemmli sample buffer. Proteins were separated by SDS-PAGE, and AP-2 was detected by Western blotting using anti-$\alpha$ adaptin antibody.

*Protein Purification*—For production of myristoylated Arf6, Bl21(DE3) bacteria were cotransformed with pET-21b-Arf6 and pBB131 containing yeast myristoyl-transferase (20). Recombinant myristoylated Arf6 with the carboxyl-terminal hexahistidine tag was prepared as previously described (21) followed by adsorption to His-bind resin (nickel-nitrilotriacetic acid-agarose; Qiagen). Thereafter, the protein was eluted with 200 mM imidazole (pH 8.0) in 10% glycerol. After gel filtration on NAP-10 columns (Amersham Biosciences), proteins were conserved in 50 mM Tris/HCl, pH 8.0; 1 mM $MgCl_2$; 1 mM dithiothreitol; 0.25 mM phenylmethylsulfonyl fluoride, and 6 $\mu$M GTP. We and others (11) have observed that the addition of a hexa-His tag does not modify the biochemical properties of Arf6 (GTP-dependent membrane binding, spontaneous and guanine nucleotide exchange factor-catalyzed nucleotide exchange activity, GAP sensitivity and effector interaction). Amino-terminally truncated (Δ13)Arf6 mutant was purified as described previously (22). Wild-type or mutant Arf6 or Arf1 GST fusion proteins were purified from bacteria according to the manufacturer's instructions (Amersham Biosciences). Purification of AP-1 and AP-2 complexes were performed essentially as described previously (23). Rat brain cytosol and P2′ fractions were prepared as described earlier (24).

*Liposome Binding Assays*—Large unilamellar vesicles of azolectin were prepared as previously described (25). Vesicles of defined composition were prepared by extrusion through a 0.4-$\mu$m pore size polycarbonate filter as described previously (26). (Isopore, Millipore). After extrusion, the sucrose-loaded vesicles were diluted five times in 20 mM Hepes/NaOH, pH 7.4 and 100 mM NaCl, centrifuged for 20 min at 400,000 × *g* (in a Beckman TLA100.3 rotor) and resuspended in the same buffer. We made a range of vesicles based on two neutral phospholipids, 40% PC and 30% PE, and as indicated in the figure legend, we added 30% PS or 30% PS + 2% (if no other indication) of phosphoinositides ($PI(3,4)P_2$, $PI(4,5)P_2$, or $PI(3,4,5)P_3$).

For the binding assays, rat brain cytosol (0.5 mg/ml) or purified AP-1 or AP-2 (0.4 $\mu$M) were incubated for 15 min at 4 °C in a 70-$\mu$l final volume of HNM buffer (50 mM Hepes/NaOH, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol) supplemented with 1 mg/ml phospholipid vesicles. To test the effect of Arf6 on the membrane recruitment of AP-2, recombinant myristoylated Arf6 or recombinant amino-terminally deleted (Δ13)Arf6 mutant (1.5 $\mu$M unless otherwise indicated) were added to the incubation. After centrifugation for 20 min at 40,000 revolutions/min (TLA 100.1 Beckman rotor) at 4 °C, the lipid pellet was resuspended in the same volume of HNM buffer. The proportion of proteins in the pellet and in the supernatant was analyzed by Sypro-Orange staining after SDS-PAGE using a fluorescence imaging system. (LAS 3000, Fujifilm).

## RESULTS AND DISCUSSION

*MyrArf6GTP Recruits Specifically the AP-2 Complex onto Phospholipid Liposomes*—By analogy with Arf1, which interacts with AP-1, -3, and -4, we studied the possibility that Arf6 recruits AP-2 to the lipid membrane. To address this question, a rat brain cytosol was incubated with liposomes in the absence or presence of recombinant Arf6 proteins. We used the GTP-loaded Arf6 wild-type and the Arf6 mutants Q67L and T44N that are respectively locked in GTP- and GDP-bound conformations (8, 13). After incubation and sedimentation of the

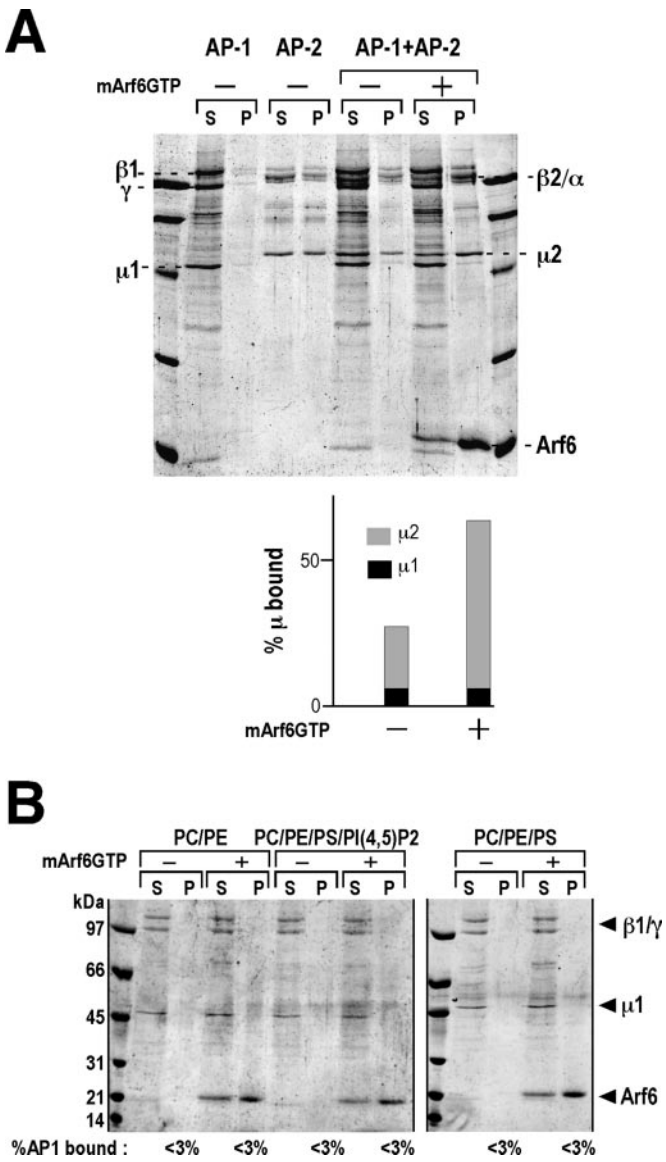


FIG. 3. **Arf6GTP recruits AP-2 but not AP-1 to liposomes.** *A*, purified AP-1 (0.8 $\mu$M) or AP-2 (0.4 $\mu$M) or a mixture of the two purified complexes were incubated with or without recombinant myrArf6GTP (1.5 $\mu$M) in the presence of PC/PE liposomes (1 mg/ml) supplemented with PS and PIP2. The vesicles were sedimented, and the protein content of supernatant (*S*) and pellet (*P*) were analyzed by SDS-PAGE and densitometry of the Sypro-Orange-stained gels. *B*, the purified AP-1 complex (0.4 $\mu$M) was incubated with or without recombinant myristoylated GTP-loaded Arf6 (*mArf6GTP*) in the presence of PC/PE liposomes, supplemented or not with PS and PIP2. Samples were analyzed as in *A*. Each experiment was repeated at least three times.

liposomes, the recruitment of AP-2 was monitored by Western blotting. Fig. 1*A* shows that AP-2 is associated to lipids only when Arf6GTP is present. This experiment indicates that Arf6GTP induces the membrane recruitment of AP-2. This could result from an Arf6-mediated increase in the synthesis of PIP2 that binds AP-2, or else from the formation of a protein complex between Arf6 and AP-2. Thus, we performed a GST pull-down experiment. We found that GST-Arf6GTP efficiently pulled down AP-2 from a detergent extract of rat brain, suggesting that the two molecules are part of the same complex (Fig. 1*B*). In contrast, neither the inactive form of Arf6 nor the two conformations of Arf1 were able to pull down the AP-2 complex (Fig. 1*B*). To look for an eventual direct interaction between Arf6 and AP-2, we performed the liposome binding experiments in presence of the purified proteins. In the absence of Arf6, purified AP-2 adaptors were almost entirely recovered in the supernatant (Fig. 1*C*). The addition of myristoylated Arf6 locked in the GDP conformation (mArf6T44N) had no effect on the membrane recruitment of AP-2. By contrast, in the presence of myristoylated GTP-loaded Arf6, a fraction of AP-2 complex was found associated with the liposomes. This membrane-bound fraction of AP-2 increased in an Arf6GTP dose-dependent manner (Fig. 1*C*). We observed that the addition of a soluble Arf6GTP, the amino-terminally deleted ($\Delta$13)Arf6 mutant, did not recruit AP-2 onto liposomes (Fig. 1*C*). This suggests that the binding of Arf6 to the membrane is required for AP-2 translocation.

Previous studies (27–29) have shown that Arf6 activates some lipid-modifying enzymes, such as the phospholipase D and the phosphatidylinositol- (4)-phosphate 5-kinase (11, 12, 30); the latter leading to the formation of phosphoinositides. Moreover, AP-2 has been shown to bind preferentially to polyphosphoinositides (31–33). Here, we analyzed the effect of different phosphoinositides on the association of AP-2 to liposomes (Fig. 2). In the absence of Arf6, we observed only a small binding of AP-2 to PI(3)P. In contrast, when PIP2 or PIP3 (2%) were incorporated into the liposomes, the binding of AP-2 was strongly increased, PI(4,5)P2 giving the highest binding (~40% of the AP-2 was associated with PIP(4,5)P2-containing vesicles). Our result confirms that AP-2 specifically recognizes polyphosphoinositides and particularly PIP(4,5)P2. In the presence of Arf6GTP, a very strong binding of AP-2 (~75%) was observed for the three types of vesicles, PI(3,4)P2 giving the lowest binding. Thus, these results show that Arf6GTP and polyphosphoinositides cooperate to recruit AP-2 to lipid membranes.

To determine whether Arf6 could also induce the membrane recruitment of AP-1, purified AP-1 and AP-2 were mixed together and incubated with liposomes in the presence or absence of Arf6GTP. After sedimentation, we observed that the AP-1 tetramer was essentially recovered in the soluble fraction independently of the presence of Arf6 (Fig. 3*A*). In contrast, Arf6 induced a strong association of AP-2 to the lipids. We tested different compositions of liposomes and did not observe any Arf6-stimulated membrane recruitment of AP-1 (Fig. 3*B*) These experiments suggest that the interaction between Arf6 and AP-2 is specific.

In conclusion, our results demonstrated that Arf6 could regulate the specific membrane recruitment of AP-2 both directly by recruiting AP-2 and indirectly by activating the PI4P5K (11).

*Arf6GTP Co-immunoprecipitates with the Clathrin Adaptor AP-2*—Next, we proceeded by conducting co-immunoprecipitation experiments in cells expressing either inactive or active Arf6 mutants. We observed that AP-2, as probed by $\alpha$-adaptin, was selectively co-immunoprecipitated with the GTP-locked mutant (Arf6Q67L) but not with the GDP-locked mutant (Arf6T44N) (Fig. 4*A*). In addition, no co-immunoprecipitation was observed between Arf6 mutants and the AP-1 complex (Fig. 4*A*). These results confirm that Arf6GTP interacts specifically with AP-2 *in vivo*.

*Expression of Arf6Q67L Leads to the Redistribution of AP-2 at the Plasma Membrane Where They Colocalize*—We then analyzed the effect of the expression of the two mutants of Arf6 on the plasma membrane distribution of AP-2. Fig. 4*B* shows a redistribution of AP-2 toward GTP-locked Arf6Q67L-enriched regions of the plasma membrane (Fig. 4*B*). On the contrary, the AP-2 localization was not affected by the expression of the GDP-locked mutant (Arf6T44N). Although one cannot exclude that the redistribution of AP-2 was due to an increase in PIP2 level, our *in vitro* experiments, as well as immunoprecipitation data, argue in favor of a direct recruitment of AP-2 by Arf6.

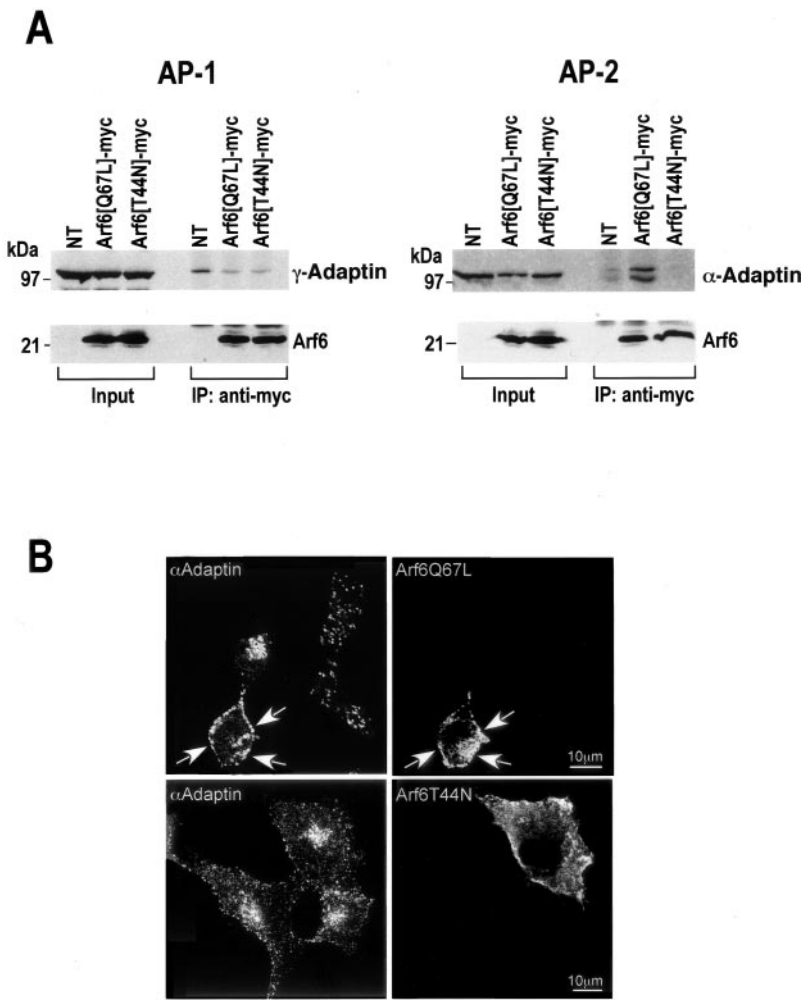


FIG. 4. **Arf6GTP but not Arf6GDP co-immunoprecipitates AP-2 and redistributes the complex at the plasma membrane.** *A*, lysates of non-transfected BHK cells (*NT*) or the transiently expressing *myc*-tagged version of Arf6Q67L (locked in GTP) or Arf6T44N (locked in GDP) were immunoprecipitated (*IP*) with an anti-*myc* antibody. Immunoprecipitates were resolved on SDS-polyacrylamide gels, transferred on nitrocellulose membranes and examined with an anti-α-adaptin antibody (*AP-2*) or with an anti-γ-adaptin (*AP-1*). 5% of the input (cell lysates) were also immunoblotted with an anti-α- or -γ-adaptin and anti-*myc* antibodies to detect the presence of endogeneous adaptor complexes and expressed Arf6 mutants, respectively. *B*, immunolocalization of α-adaptin in BHK cells expressing HA-tagged Arf6Q67L (Arf6GTP) or Arf6T44N (Arf6GDP).

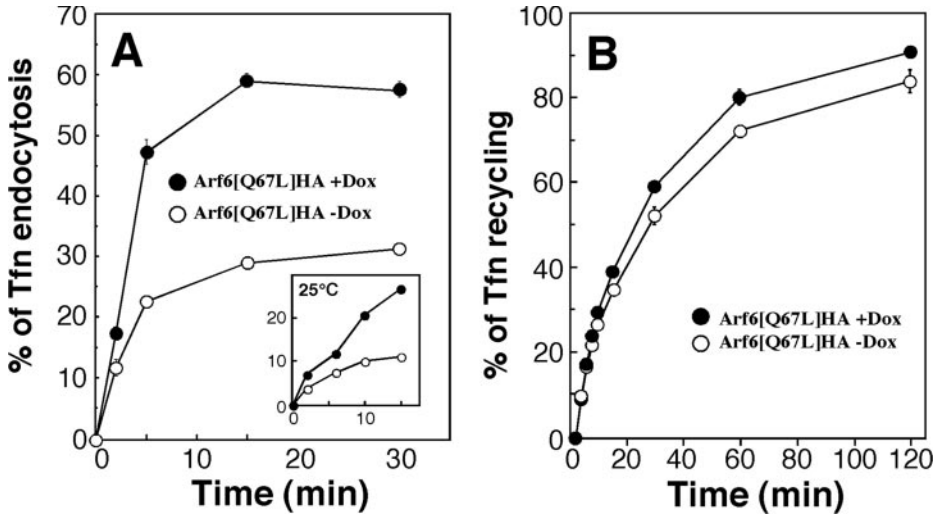


FIG. 5. **Arf6 is involved in Tfn internalization.** *A*, kinetics analysis of [$^{125}$I]Tfn endocytosis on HeLa cells expressing (−Dox, ●) or not (+Dox, ○) Arf6(Q67L)-HA at 37 or 25 °C (*inset*) for the indicated times. *B*, recycling of Tfn is shown with Arf6(Q67L)-HA cells expressing (−Dox, ●) or not (+Dox, ○) the mutant, which were pulsed with [$^{125}$I]Tfn for 10 min at 37 °C. The cells were then incubated at 25 °C for the indicated times to allow the recycling of internalized [$^{125}$I]Tfn. Measurements are plotted as the percentage of total [$^{125}$I]Tfn (*i.e.* the sum of intracellular and cell-bound cpm for internalization and the sum of the cpm recovered in the medium and cell-bound for recycling). *Errors bars* represent the range in duplicate experiments.

*Expression of Arf6Q67L Strongly Inhibits the Internalization of the Tfn-R but Has No Effect on Its Recycling*—Whether Arf6 regulates clathrin-dependent or -independent endocytosis is still a controversial question. Our findings shed new light on this question. Because Arf6GTP interacts with AP-2 both *in vitro* and *in vivo* and recruits the complex to the membrane, we proposed that Arf6 could control clathrin-mediated endocytosis. To test this hypothesis, we examined the trafficking of the Tfn-R as a marker for clathrin-mediated endocytosis. To avoid artifacts associated with overexpression of the Tfn-R, as well as Arf6 proteins, we prepared stable HeLa cell lines expressing the GTPase-defective mutant of Arf6 under the tetracycline-regulated repressible system and monitored the trafficking of the endogenous Tfn-R by following iodinated Tfn. Fig. 5*A* shows that expression (−Dox) of Arf6Q67L strongly decreased Tfn internalization. To slow down and enhance characterization of the initial rate, the experiment was also conducted at 25 °C (Fig. 5*A*, *inset*). The initial rate was 9.5 ± 0.4%/min in control cells (+Dox) and 4.58 ± 0.04%/min in Arf6Q67L-expressing cells (−Dox). After 15 min, the plateau reached was reduced by

45.4 ± 1.5% in Arf6Q67L-expressing cells. These results demonstrated that overexpression of Arf6GTP interferes with endogenous Tfn-R endocytosis. This Arf6Q67L-induced inhibition of Tfn-R endocytosis was probably not a consequence of the overactivation of the Arf6 effector PI4P5Kinase. Indeed, it has been demonstrated recently that overexpressing the kinase leads to a stimulation of Tfn-R internalization (34). An explanation for this Arf6-induced inhibition could be that when Arf6 is involved in clathrin-dependent endocytosis by recruiting AP-2, the expression of a constitutively activated Arf6 should compete with the endogenous Arf6 for AP-2 binding at the plasma membrane. Such a competition would lead to the inhibition of the AP-2-dependent internalization as observed.

We next analyzed the role of Arf6 on Tfn recycling. [$^{125}$I]Tfn was internalized for 10 min at 37 °C. The cells were then cooled down on ice and acid-stripped to remove the cell surface-associated Tfn. The cells were then incubated at 25 °C to allow [$^{125}$I]Tfn recycling. The experiment was performed at 25 °C to slow down the reaction, because as observed for the internalization at 37 °C, the recycling was fast, and the initial rate was difficult to measure. As shown in Fig. 5*B*, we measured a slight inhibition when cells expressed the Arf6Q67L mutant. The initial rate of Tfn recycling was 2.99 ± 0.07%/min in control cells (+Dox) and 2.68 ± 0.08%/min in Arf6Q67L-expressing cells (−Dox). At the plateau, [$^{125}$I]Tfn recycling was slightly inhibited (≤10%) in Arf6Q67L-expressing cells.

These results demonstrate that Arf6 is primarily involved in the first steps of Tfn-R endocytosis and has only a slight, although reproducible, effect on its recycling. Taken altogether, our results suggest that Arf6 plays a role in endocytosis by controlling the assembly of the AP-2/clathrin coat. Thus, Arf family proteins appear to be widely used by the intracellular vesicular machinery to control membrane association of coat proteins. However, cellular depletion of Arf6, by small interfering RNA, has recently been shown to affect not only the clathrin-dependent but also clathrin-independent ligand-induced endocytosis of a set of G-protein-coupled receptors (35). These results suggest that Arf6 may exert an additional role in endocytosis than just recruiting the AP-2 complex. This has been demonstrated at least in polarized cells where Arf6GTP was involved in the dynamin-mediated endocytosis of the E-cadherin by recruiting the nucleoside diphosphate-kinase Nm23 protein (6). Thus, it appears that by regulating different effectors, Arf6 could control different endocytic pathways as well as different steps of the same pathway. Further experiments will now be necessary to define the molecular mechanism that couples the activation of Arf6 with the receptor to be internalized.

*Acknowledgments*—We are indebted to W. Boll and I. Rapoport for help with adaptor protein purification. We thank Drs. K. Singer, M. Chabre, P. Chavrier, and B. Antonny for critical reading of the manuscript.

REFERENCES

1. Nie, Z., Hirsch, D. S., and Randazzo, P. A. (2003) *Curr. Opin. Cell Biol.* **15,** 396–404
2. McMahon, H. T., and Mills, I. G. (2004) *Curr. Opin. Cell Biol.* **16,** 379–391
3. Chavrier, P., and Goud, B. (1999) *Curr. Opin. Cell Biol.* **11,** 466–475
4. Altschuler, Y., Liu, S., Katz, L., Tang, K., Hardy, S., Brodsky, F., Apodaca, G., and Mostov, K. (1999) *J. Cell Biol.* **147,** 7–12
5. Franco, M., Peters, P. J., Boretto, J., van Donselaar, E., Neri, A., D'Souza-Schorey, C., and Chavrier, P. (1999) *EMBO J.* **18,** 1480–1491
6. Palacios, F., Schweitzer, J. K., Boshans, R. L., and D'Souza-Schorey, C. (2002) *Nat. Cell Biol.* **11,** 11
7. Blagoveshchenskaya, A. D., Thomas, L., Feliciangeli, S. F., Hung, C. H., and Thomas, G. (2002) *Cell* **111,** 853–866
8. D'Souza-Schorey, C., Li, G., Colombo, M. I., and Stahl, P. D. (1995) *Science* **267,** 1175–1178
9. Radhakrishna, H., and Donaldson, J. G. (1997) *J. Cell Biol.* **139,** 49–61
10. Caumont, A. S., Galas, M. C., Vitale, N., Aunis, D., and Bader, M. F. (1998) *J. Biol. Chem.* **273,** 1373–1379
11. Krauss, M., Kinuta, M., Wenk, M. R., De Camilli, P., Takei, K., and Haucke, V. (2003) *J. Cell Biol.* **162,** 113–124
12. Aikawa, Y., and Martin, T. F. (2003) *J. Cell Biol.* **162,** 647–659
13. Macia, E., Luton, F., Partisani, M., Cherfils, J., Chardin, P., and Franco, M. (2004) *J. Cell Sci.* **117,** 2389–2398
14. Kirchhausen, T. (2002) *Cell* **109,** 413–416
15. Boehm, M., and Bonifacino, J. S. (2001) *Mol. Biol. Cell* **12,** 2907–2920
16. Traub, L. M. (2003) *J. Cell Biol.* **163,** 203–208
17. Conner, S. D., and Schmid, S. L. (2003) *Nature* **422,** 37–44
18. Franco, M., Boretto, J., Robineau, S., Monier, S., Goud, B., Chardin, P., and Chavrier, P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9926–9931
19. Breitfeld, P. P., Harris, J. M., and Mostov, K. E. (1989) *J. Cell Biol.* **109,** 475–486
20. Duronio, R. J., Jackson-Machelski, E., Heuckeroth, R. O., Olins, P. O., Devine, C. S., Yonemoto, W., Slice, L. W., Taylor, S. S., and Gordon, J. I. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1506–1510
21. Chavrier, P., and Franco, M. (2001) *Methods Enzymol.* **329,** 272–279
22. Macia, E., Chabre, M., and Franco, M. (2001) *J. Biol. Chem.* **276,** 24925–24930
23. Campbell, C., Squicciarini, J., Shia, M., Pilch, P. F., and Fine, R. E. (1984) *Biochemistry* **23,** 4420–4426
24. Huttner, W. B., Schiebler, W., Greengard, P., and De Camilli, P. (1983) *J. Cell Biol.* **96,** 1374–1388
25. Franco, M., Chardin, P., Chabre, M., and Paris, S. (1993) *J. Biol. Chem.* **268,** 24531–24534
26. Macia, E., Paris, S., and Chabre, M. (2000) *Biochemistry* **39,** 5893–5901
27. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) *Cell* **75,** 1137–1144
28. Cockcroft, S., Thomas, G. M., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O., and Hsuan, J. J. (1994) *Science* **263,** 523–526
29. Massenburg, D., Han, J. S., Liyanage, M., Patton, W. A., Rhee, S. G., Moss, J., and Vaughan, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 11718–11722
30. Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Kanaho, Y. (1999) *Cell* **99,** 521–532
31. Gaidarov, I., Chen, Q., Falck, J. R., Reddy, K. K., and Keen, J. H. (1996) *J. Biol. Chem.* **271,** 20922–20929
32. Rohde, G., Wenzel, D., and Haucke, V. (2002) *J. Cell Biol.* **158,** 209–214
33. Collins, B. M., McCoy, A. J., Kent, H. M., Evans, P. R., and Owen, D. J. (2002) *Cell* **109,** 523–535
34. Padron, D., Wang, Y. J., Yamamoto, M., Yin, H., and Roth, M. G. (2003) *J. Cell Biol.* **162,** 693–701
35. Houndolo, T., Boulay, P. L., and Claing, A. (2005) *J. Biol. Chem.* **280,** 5598–5604